Italian Botanist 18: 29–50 (2024)
doi: 10.3897/italianbotanist.18.123376
https://italianbotanist.pensoft.net

RESEARCH ARTICLE

Italian Botanist
Published by Società Botanica Italiana

In vitro propagation of the orchid *Phalaenopsis circus via* organogenesis and somatic embryogenesis using protocorm and thin cell layer explants

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Academic editor: Stefania Biondi | Received 18 March 2024 | Accepted 29 April 2024 | Published 27 September 2024

Citation: Kiaheirati H, Hashemabadi D, Kaviani B (2024) *In vitro* propagation of the orchid *Phalaenopsis circus via* organogenesis and somatic embryogenesis using protocorm and thin cell layer explants. Italian Botanist 18: 29–50. https://doi.org/10.3897/italianbotanist.18.123376

Abstract

Orchids of the genus *Phalaenopsis* have high economic value in the floriculture industry and market and high potential for breeding programs. *In vitro* propagation makes it possible to clonally mass proliferate and conserve this valuable plant. In the current research, efficient protocols, some reported for the first time, for *in vitro* propagation of *Phalaenopsis circus* through organogenesis and somatic embryogenesis (SE) are presented. We used protocorms obtained from seeds and thin cell layers (TCLs) prepared from leaves as explants. Explants were cultured on Murashige and Skoog (MS) basal medium enriched with various concentrations and combinations of plant growth regulators. Protocorms were cultured on media fortified with 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with N-phenyl-N′-1,2,3-thiadiazol-5-yl-urea (TDZ), and α-naphthalene-acetic acid (NAA) in combination with N^6-furfuryl adenine or kinetin (Kin) for organogenesis, as well 2,4-D in combination with NAA for SE. These protocorms produced either protocorm-like bodies (PLBs) or somatic embryos. Results showed that the highest number of PLBs (75.0) was obtained on medium enriched with 1.0 mg l^{-1} 2,4-D. Maximum number of somatic embryos (12.3/explant) was obtained on medium containing 0.5 mg l^{-1} 2,4-D together with 2.0 mg l^{-1} NAA. The use of transversal TCLs with 2–3 cell layers as explants cultured on medium supplemented with 0.5 mg l^{-1} IBA combined with 1.0 mg l^{-1} TDZ produced the highest number of plantlets. Plantlets were transferred to pots and acclimatized in ambient greenhouse conditions with 100% survival rate.

Keywords

Epiphytic orchids, orchid propagation, organogenesis, plant growth regulators, somatic embryogenesis, thin cell layers

Introduction

Orchids (family Orchidaceae) are among the most diverse of the flowering plant families, with more than 800 genera and 25,000 species (Chugh et al. 2009). *Phalaenopsis* as a cut and pot flowering plant is one of the most popular orchids in the world, through the development of many artificial hybrids. The genus *Phalaenopsis* comprises approximately 60 species native to tropical rainforests of South and South-East Asia, Australia and New Guinea (Winkelmann et al. 2006). They are epiphytic plants, and consist of only a few leathery leaves (Sinha et al. 2010).

Natural clonal propagation of orchids is a slow process, which results in traits segregation and is, therefore, not possible for *Phalaenopsis*. Also, sexual propagation of orchids leads to the production of heterozygous plants. Therefore, establishment of protocols for *in vitro* propagation of orchids is important as an alternative procedure for high frequency regeneration of these plants. Many orchid species are vulnerable, rare and/or threatened. One of the important approaches for conservation of these plants is *in vitro* propagation. *In vitro* propagation is an extremely useful technique for clonal propagation of many species, particularly ornamental plants like orchids (Guo et al. 2024). *In vitro* techniques may also be applied for production of a large number of healthy and disease-free plantlets in a short span of time, allowing changes in growth parameters, and generation of propagules all year round (Pati et al. 2006; Engelmann 2011). Large-scale propagation of orchids using tissue culture techniques has meant that orchids occupy a position as one of the top ten cut and pot flowers (Chugh et al. 2009). *In vitro* propagation of orchids has a few problems like low rate of shoot multiplication, high cost of production, poor rooting frequency, exudation of phenolic compounds from explants, transplantation to field, and somaclonal variation (Chugh et al. 2009; Bhattacharyya et al. 2016).

Protocorms of orchids are derived from the seed. Protocorm-like bodies (PLBs), on the other hand, are derived from vegetative organs like leaves, stems and protocorms during *in vitro* culture. Seed-derived protocorms may also be used to induce somatic embryogenesis (SE). Since the general characteristics of growth and structure are similar to those of protocorms, the regenerated structure is termed PLB (Lee et al. 2013). Therefore, PLBs are similar to protocorms in morphology and biological characteristics (Lee et al. 2013; Cardoso et al. 2020). The main difference between protocorms and PLBs is basically the origin of the tissue. PLBs are able to differentiate a shoot apical meristem and then a complete plantlet. Induction of PLBs and somatic embryo differentiation (directly or indirectly) facilitate the micropropagation of orchids (Chen et al. 2019). In orchids, the formation of protocorms and PLBs is regulated by various factors, and plant growth regulators (PGRs) are among the most important ones (Cardoso et al. 2020). N-phenyl-N′-1,2,3-thiadiazol-5-yl-urea (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D) are the most suitable PGRs for callus and PLB induction, as reported for *Cymbidium*, *Phalaenopsis*, *Dendrobium*, and *Paphiopedilum* (Cardoso et al. 2020; Guo et al. 2024).

In vitro propagation of genera belonging to the Orchidaceae family including *Phalaenopsis* has been reported using various explants such as leaf segments (foliar explants), node sections, rhizome segments, root segments, protocorms, PLBs, tubers, shoot tips,

flower buds, and inflorescence axes (Chugh et al. 2009; Sinha et al. 2010; Roy et al. 2011; Panwar et al. 2012; Zeng et al. 2012; Baker et al. 2014; Mahendran, 2014; Chen et al. 2015; Bhattacharyya et al. 2016; Kaviani et al. 2017; Asa et al. 2019; Mohammadi et al. 2019). Among all these explants, PLBs and protocorms are more efficient because of rapid multiplication on solid or liquid culture media, and maximum production in a short period of time (Luo et al. 2003; Roy et al. 2011; Zeng et al. 2012).

Many protocols for *in vitro* propagation of orchids using PLBs and protocorms as explants and various PGRs have been reported (Sinha et al. 2010; Baker et al. 2014; Kaviani et al. 2017; Mohammadi et al. 2019; Zakizadeh et al. 2019; Asa et al. 2020; Guo et al. 2024). *In vitro* propagation through PLBs obtained from somatic tissues is an important approach to obtain genetically stable plants and improvement of quality. An efficient *in vitro* propagation method for *Phalaenopsis* sp. using PLBs derived from leaf explants was introduced by Park et al. (2002b). Ishii et al. (1998), Kuo et al. (2005) and Chen and Chang (2006) proposed a protocol for regeneration of a *Phalaenopsis* cultivar by direct SE starting from leaf explant. Medium composition for tissue culture of orchids by PLBs and protocorms is species-specific and depends on several factors particularly PGRs (Luo et al. 2009; Guo et al. 2024).

PGRs such as α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), TDZ, 6-benzyladenine (BA) and 6-furfurylaminopurine or kinetin (Kin) have been used for *in vitro* propagation of *Phalaenopsis* orchids through organogenesis and SE (Tanaka 1992; Ernst 1994; Tokuhara and Mii 2001; Park et al. 2002a, b; Kuo et al. 2005; Sinha et al. 2007; Kaviani et al. 2017; Asa et al. 2019; Mohammadi et al. 2019). In *Phalaenopsis amabilis*, TDZ promoted direct SE from the epidermal cells of leaf explants (Chugh et al. 2009). Embryo formation on leaf explants was retarded by auxins like indole-3-acetic acid (IAA), IBA, NAA, and 2,4-D but promoted by cytokinins like 2-isopentyladenine (2-iP), zeatin (Zt), Kin, BAP and TDZ (Chen and Chang 2001).

Plant regeneration from thin cell layers (TCLs) is a simple and effective technique for clonal *in vitro* propagation of orchids (Teixeira da Silva 2013). It is the most important technique for *in vitro* propagation of some species. This system consists of explants of a small size (approximately 0.5–1.0 mm thick) excised from different organs (stem, leaf, flower, cotyledons, hypocotyl, epicotyl, apical meristem or embryo), either longitudinally (explants comprise epidermal, cortical, cambium, perivascular and medullary tissue, and parenchyma cells) or transversally (explants comprise all tissues present in the organ) (Chugh et al. 2009). TCLs have been successfully applied for protocorm, PLB, floral stalk-derived leaf, shoot, and callus induction in *Aranda* (Lakshmanan et al. 1995), *Coelogyne cristata* (Naing et al. 2011), *Cymbidium* spp. (Begum et al. 1994; Nayak et al. 2002; Malabadi et al. 2008; Hossain et al. 2010; Vyas et al. 2010), *Dendrobium* spp. (Zhao et al. 2007; Rangsayatorn 2009; Jaiphet and Rangsayatorn 2010), *Doritaenopsis* (Park et al. 2000), *Paphiopedilum* (Liao et al. 2011), *Renanthera* (Wu et al. 2012), *Spathoglottis* (Teng et al. 1996), and *Xenikophyton* (Mulgund et al. 2011). NAA, BAP, and TDZ are some of the PGRs used for *in vitro* propagation using TLCs through organogenesis and SE.

The purpose of the current research was to evaluate the effect of different concentrations of Kin, TDZ, NAA, IBA and 2,4-D, individually and in combination on

in vitro propagation of *P. circus via* organogenesis and SE using two types of explants: protocorms obtained from seed and TCLs obtained from vertically cut leaves. Both organogenesis and SE can be direct and indirect (*via* callus). The difference in the type of explant and the type, concentration, and combination of PGRs play an important role in obtaining different results.

Materials and methods

Explants source and sterilization for organogenesis and SE

Capsules of *Phalaenopsis circus* were isolated from the flowers of plants grown in the greenhouse of the Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Iran. The approximate age of these capsules was 150 days. The capsules were first washed under running tap water along with a few drops of dishwashing liquid for 30 min and rinsed thoroughly with distilled water. Then, they were surface sterilized in 50% sodium hypochlorite solution containing 5% active chloride for 20 min with a drop of Tween 20, and washed thoroughly in distilled water and finally dipped in 70% alcohol for a minute and flamed. The sterilized capsules were cut longitudinally with the help of a sharp sterilized surgical blade for extracting seeds. Seeds were inoculated on MS (Murashige and Skoog 1962) medium for production of protocorms. Media were supplemented with 3% sucrose and solidified with 0.8% agar. Media were adjusted to pH 5.6–5.8 with 1 N HCl or NaOH before autoclaving at 121 °C, 105 kPa for 20 min. Following establishment, all the cultures were incubated at 24 ± 2 °C, 70–80% RH, and 16-h photoperiod of 50–60 µmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes.

Plantlet formation from protocorms and PLBs through organogenesis and SE

The explants (protocorms obtained from germinated seeds) were cultured on MS medium containing 3% sucrose and 0.8% agar. The pH of the media was adjusted to 5.6–5.8 with 0.1 N NaOH or HCl prior to autoclaving. All media contained in culture bottles were autoclaved at 105 kPa and 121 °C for 20 min. The media were enriched with different concentrations of TDZ (0.0, 0.1, 1.0, 2.0 and 5.0 mg l^{-1}) and 2,4-D (0.0, 0.01, 0.1, 1.0 and 2.0 mg l^{-1}), or with NAA (0.0, 0.1, 0.5, 1.0 and 5.0 mg l^{-1}) and Kin (0.0, 0.5, 1.0, 2.0 and 5.0 mg l^{-1}), either individually or in combination for organogenesis. For induction of somatic embryos, media were fortified with different concentrations of NAA (0.0, 0.1, 0.5, 1.0 and 5.0 mg l^{-1}) and 2,4-D (0.0, 0.01, 0.1, 0.5 and 1.0 mg l^{-1}), individually or in combination.

TCLs source and sterilization

Leaves of *P. circus* were detached from *ex vitro*-grown plantlets for preparation of TCLs. Leaves were washed for 3–4 h under running tap water and rinsed thoroughly with distilled water. Then, they were surface sterilized in 50% sodium hypochlorite solu-

tion containing 2.5% active chloride for 15 min with a drop of Tween 20, and washed thoroughly in distilled water followed by dipping in 10% silver nano-particles. Finally, explants were dipped in 70% alcohol for a minute followed by rinsing with sterilized distilled water. The sterilized leaves were cut vertically as segments in diameters of 0.5–1.0 mm and used as explants (TCLs). TCLs were cultured on MS medium supplemented with 3% sucrose and solidified with 0.8% agar. The media were enriched with different concentrations of TDZ (0.0, 0.01, 0.1, 0.5 and 1.0 mg l^{-1}) and IBA (0.0, 0.1, 0.5 and 1.0 mg l^{-1}), either individually or in combination for plantlets regeneration through organogenesis. Media were adjusted to pH 5.6–5.8 before autoclaving at 121 °C, 105 kPa for 20 min. All the cultures were incubated at 24 ± 2 °C, 70–80% RH, and a 16 h/8 h day/night photoperiod with an irradiance of 50–60 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes.

General scheme of the experiments

The present study was carried out in two sections: organogenesis and SE. First of all, seeds were sown on MS medium without PGRs for production of protocorms. The protocorms were then cultured on media containing different concentrations of TDZ and 2,4-D (for organogenesis), Kin and NAA (for organogenesis), as well NAA and 2,4-D (for SE). On the other hand, TCLs obtained from leaves were cultured on MS media containing different concentrations of IBA and TDZ (for organogenesis). Most plantlets were produced through direct organogenesis and SE (without callus production). Normally, organogenesis consists in the regeneration of shoots (stem with leaves) which are then induced to root after transfer to a new medium. Sometimes, both shoots and roots are produced on the same medium. SE, instead, always produces a complete plantlet.

Measured parameters.

After 60 days, PLB number, leaf length, leaf number, root length, and root number (produced through organogenesis) were measured. The number of somatic embryos, leaf number, root length, and root number (produced through SE) were also measured. Plantlet number, leaf length, leaf number, root length, and root number produced through direct organogenesis on TCLs were also recorded.

Plantlet development and acclimatization

For *ex vitro* establishment, *in vitro* well-rooted plantlets were taken out from culture vessels and washed thoroughly with sterile distilled water to remove adherent medium from the plantlet body and transferred to plastic pots (18 cm height × 12 cm diameter) filled with a potting mixture of LECA (Light Expanded Clay Aggregate), peat moss, coco peat, charcoal soil, coco chips and perlite in the proportion of 15:10:20:5:30:20%. All the pots were then transferred to a greenhouse with temperature of 24 ± 2ºC to 20 ± 2ºC day/night (light intensity of 3,500 lux, RH of 80–90% and a 14 h/10 h day/

night photoperiod) for acclimatization. The pots were covered with another plastic pot (18 cm height × 12 cm diameter) to retain moisture. These pots were removed after two weeks. The number of surviving plants was recorded after three months of transfer.

Experimental design and data analysis

The experiments were established in a completely randomized design. For each treatment, three replicates and for each replicate, three specimens (explants) were taken (in total 95 treatments, 285 replicates, and 855 explants). PGR-free MS medium was used as control in the experiments. Data were subjected to analysis of variance (ANOVA) and means were compared by the LSD test at $P < 0.05$ using the SPSS ver. 17 (SPSS Inc., USA).

Results

Effect of 2,4-D and TDZ on *in vitro* propagation through organogenesis

PLBs were produced from protocorms and then developed into plantlets on most media. There were statistically significant differences ($p \leq 0.01$) in PLB number as well leaf length among different concentrations of 2,4-D in combination with TDZ (Table 1). Maximum PLB number (75.0) was obtained in explants treated with 1.0 mg l^{-1} 2,4-D without TDZ (Table 2). Increasing 2,4-D and TDZ concentrations had no positive effect on PLB number (Table 2). The minimum PLB number (14.67) was obtained in explants treated with 5.0 mg l^{-1} TDZ without 2,4-D (Table 2). All treatments without the presence of 2,4-D induced lower PLB production (<18; Table 2). The highest average leaf length (5.23 cm/explant) was obtained with 2.0 mg l^{-1} 2,4-D without TDZ (Fig. 1). The lowest average root length (2.0 cm/explant) was observed in control explants.

Effect of NAA and Kin on *in vitro* propagation through organogenesis

Differences in PLB number, leaf length and root length in samples grown under different concentrations of Kin in combination with NAA were significant ($p \leq 0.01$) (Table 1). PLB production was strongly influenced by external addition of PGRs (NAA together with Kin). Varied responses in terms of PLB number and leaf and root length were obtained using different concentrations of NAA and Kin. When the explants were grown on medium supplemented with 1.0 mg l^{-1} Kin in combination with 0.5 mg l^{-1} NAA, maximum number of PLBs (63.0) was produced. Treatment containing 0.5 mg l^{-1} Kin in combination with 0.5 mg l^{-1} NAA was found to be the next most effective PGR combination affecting the production of PLBs (51.0), followed by 0.5 mg l^{-1} NAA together with 2.0 mg l^{-1} Kin (49.67 PLBs; Table 3). When the explants were grown on control medium, minimum number of PLBs (13.67) was

Table 1. Analysis of variance of the effect of different concentrations of 2,4-D, NAA, IBA, Kin and TDZ on measured parameters of *Phalaenopsis circus* cultured under *in vitro* organogenesis and SE conditions.

Source of variations	df	Mean squares				
		PLB number	Leaf length	Leaf number	Root length	Root number
2,4-D	4	2482.51**	6.12**	1.61ns	6.82**	21.49**
TDZ	4	123.95**	0.38ns	27.81**	0.17ns	0.45ns
2,4-D × TDZ	16	186.55**	0.69**	0.36ns	0.23ns	0.67ns
Error	48	22.91	0.24	0.66	0.15	0.9
CV (%)	-	12.65	17.08	20.67	13.21	32.85
NAA	4	921.49**	0.86**	3.19*	13.76**	16.75**
Kin	4	843.79**	5.74**	2.49ns	1.01**	1.15ns
NAA × Kin	16	119.58**	1.08**	1.63ns	1.26**	1.01ns
Error	48	37.99	0.18	0.996	0.17	0.78
CV (%)	-	19.12	16.24	31.45	12.41	24.51
		Plantlet number				
IBA	3	12.59**	1.03**	8.82**	13.59**	1.62ns
TDZ	4	10.23**	9.97**	1.44ns	0.48**	4.07**
IBA × TDZ	12	3.57**	0.15ns	1.89*	1.02**	2.12**
Error	38	1.02	0.21	0.81	0.12	0.65
CV (%)	-	24.9	14.55	26.65	11.45	27.29
		Somatic embryo number				
2,4-D	4	58.367**	-	2.147*	4.530**	8.453**
NAA	4	2.567ns	-	22.113**	4.556**	0.687ns
2,4-D × NAA	16	3.892*	-	0.480ns	0.91**	1.02ns
Error	48	1.928	-	0.702	0.224	0.57
CV (%)	-	20.22	-	26.62	14.49	28.74

*, **: Significant at the 0.05 and 0.01 probability level, respectively, ns: Not significant at p=0.05, df: degree of freedom, CV: coefficient of variations.

produced. The highest average leaf length (5.03 cm/explant) was measured with 0.1 mg l^{-1} NAA together with 2.0 mg l^{-1} Kin (Fig. 2). The lowest average leaf length (1.6 cm) was observed in controls (without PGRs). The highest average root length (6.87 cm/explant) was obtained with 1.0 mg l^{-1} NAA together with 0.5 mg l^{-1} Kin. The lowest average root length (1.77 cm) was obtained in explants treated with 0.5 mg l^{-1} Kin without NAA.

Effect of 2,4-D and NAA on *in vitro* propagation through SE

The data clearly show that there is a significant difference among different concentrations of NAA and 2,4-D for somatic embryos number ($p \leq 0.05$) and root length ($p \leq 0.01$) (Table 1). Mean comparison of the data showed that when the explants were treated with 0.5 mg l^{-1} 2,4-D together with 2.0 mg l^{-1} NAA and 0.5 mg l^{-1} 2,4-D together with 0.1 mg l^{-1} NAA, somatic embryos number/explant (12.33 and 12.00, respectively) was maximum (Table 4, Fig. 3). The lowest somatic embryos number (4.33) was observed in explants treated with 0.1 mg l^{-1} 2,4-D together with 0.1 mg l^{-1} NAA. The highest average root length (4.97 cm/explant) was obtained with 0.1 mg l^{-1}

Table 2. Mean comparison of the effect of different concentrations of 2,4-D and TDZ on measured parameters of *Phalaenopsis circus* cultured under *in vitro* organogenesis conditions.

	Mean comparison	
2,4-D + TDZ (mg l^{-1})	**PLB number**	**Leaf length**
0.0 + 0.0	18.0h	2.0h
0.0 + 0.1	16.67h	2.4e-h
0.0 + 1.0	17.0h	2.07gh
0.0 + 2.0	16.0h	2.33fgh
0.0 + 5.0	14.67h	2.23fgh
0.01 + 0.0	33.0g	2.27fgh
0.01 + 0.1	37.0efg	2.5d-h
0.01 + 1.0	40.0d-g	2.77d-h
0.01 + 2.0	39.0d-g	2.53d-h
0.01 + 5.0	44.33cde	2.87d-g
0.1 + 0.0	45.33bcd	2.57d-h
0.1 + 0.1	42.33c-f	2.8d-h
0.1 + 1.0	52.67b	2.7d-h
0.1 + 2.0	40.0d-g	2.63d-h
0.1 + 5.0	41.67c-f	2.43d-h
1.0 + 0.0	75.0a	2.83d-g
1.0 + 0.1	52.67b	3.17cde
1.0 + 1.0	49.33bc	2.63d-h
1.0 + 2.0	39.0d-g	3.23cd
1.0 + 5.0	36.33fg	2.9def
2.0 + 0.0	39.67d-g	5.23a
2.0 + 0.1	36.0fg	4.4b
2.0 + 1.0	36.33fg	3.73bc
2.0 + 2.0	40.0d-g	3.2cde
2.0 + 5.0	44.33cde	2.9def

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

2,4-D together with 2.0 mg l^{-1} NAA. Root length (4.93 and 4.70 cm) was highest in explants treated with 1.0 mg l^{-1} 2,4-D together with 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} 2,4-D without NAA, respectively (Table 4). The lowest average root length (2.30 cm) was measured in control explants.

Effect of IBA and TDZ on *in vitro* propagation through TCLs

Plantlets were produced from TCLs through direct organogenesis on some media. Statistically significant differences were observed between the means for plantlet number, root length and root number ($p\leq0.01$), as well leaf number ($p\leq0.05$) and applied PGRs (IBA together with TDZ) (Table 1). The highest number of plantlets (9.00) was counted in explants grown on media enriched with 0.5 mg l^{-1} IBA together with 1.0 mg l^{-1} TDZ. This number was much higher than those observed with other concentrations of IBA and TDZ, applied alone or in combination (Table 5). The lowest number of leaves (2.70) was obtained in control explants (Fig. 4). The application of 0.5 mg l^{-1} IBA along with 1.0 (Fig. 4) and 0.5 mg l^{-1} is optimum for leaves production.

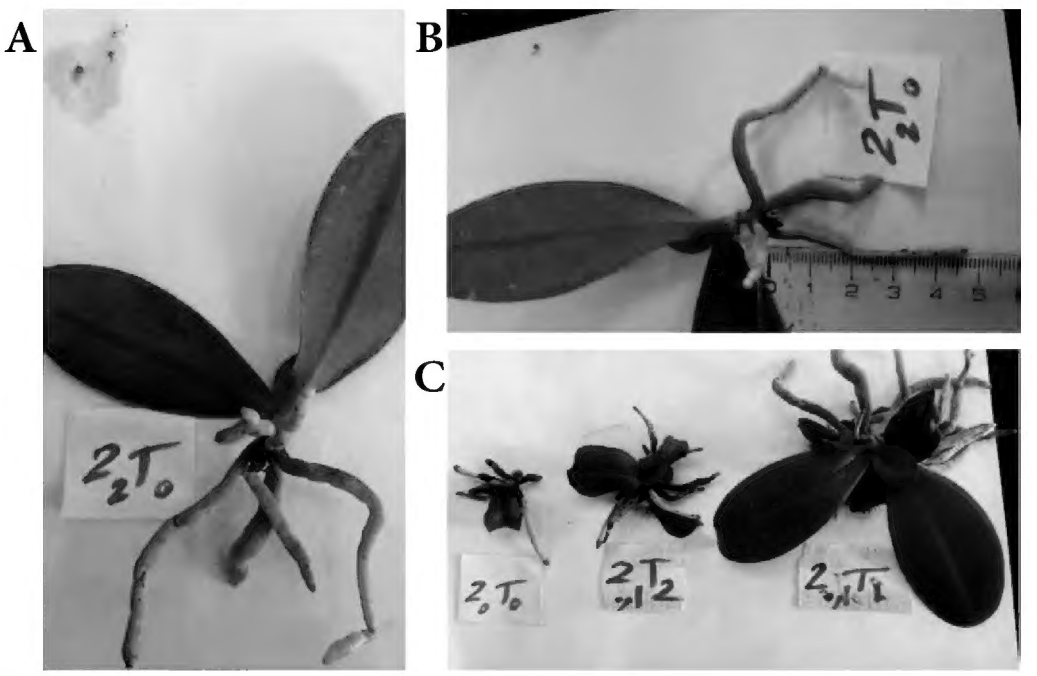

Figure 1. Micropropagation process of *Phalaenopsis circus* using different concentrations of 2,4-D and TDZ through direct organogenesis. Growth and development of leaves and roots from cultured protocorm explants obtained from germinated seeds **A, B** on medium enriched with 2.0 mg l^{-1} 2,4-D without TDZ **C** control (left), 0.1 mg l^{-1} 2,4-D and 2.0 mg l^{-1} TDZ (middle), and 0.1 mg l^{-1} 2,4-D and 1.0 mg l^{-1} TDZ (right). Scale bar: 10 mm.

Table 3. Mean comparison of the effect of different concentrations of NAA and Kin on measured parameters of *Phalaenopsis circus* grown under *in vitro* conditions for organogenesis.

	Mean comparison		
NAA + Kin (mg l^{-1})	**PLB number**	**Leaf length (cm)**	**Root length (cm)**
0.0 + 0.0	13.67j	1.6g	2.23j-l
0.0 + 0.5	22.33hij	2.13c-g	1.77l
0.0 + 1.0	33.0d-g	2.73cd	2.53h-n
0.0 + 2.0	31.0de-i	3.93b	2.2j-l
0.0 + 5.0	21.33ij	2.37c-f	2.07K-l
0.1 + 0.0	21.33ij	2.07d-g	2.4i-l
0.1 + 0.5	38.67cd	2.7cd	3.07e-i
0.1 + 1.0	48.0bc	2.57cde	2.8f-j
0.1 + 2.0	38.33cd	5.03a	3.27efg
0.1 + 5.0	23.33 g-j	2.43c-f	3.07e-i
0.5 + 0.0	25.67f-i	2.0efg	3.2e-h
0.5 + 0.5	51.0b	2.57cde	3.13e-h
0.5 + 1.0	63.0a	2.8c	3.57de
0.5 + 2.0	49.67b	3.9b	2.67g-n
0.5 + 5.0	31.0d-i	2.0efg	3.3efg
1.0 + 0.0	23.67g-j	1.67g	4.93b
1.0 + 0.5	28.0e-i	2.37c-f	6.87a
1.0 + 1.0	27.33e-i	3.6b	4.4bc
1.0 + 2.0	24.33ghi	2.17c-g	4.23cd
1.0 + 5.0	26.67f-i	1.87fg	3.47ef
5.0 + 0.0	30.33d-i	1.83fg	3.43ef
5.0 + 0.5	32.33d-h	2.47c-f	3.73cde
5.0 + 1.0	35.67def	2.67cde	3.20e-h
5.0 + 2.0	37.0de	2.5c-f	3.73cde
5.0 + 5.0	29.33d-i	2.73cd	4.20f

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

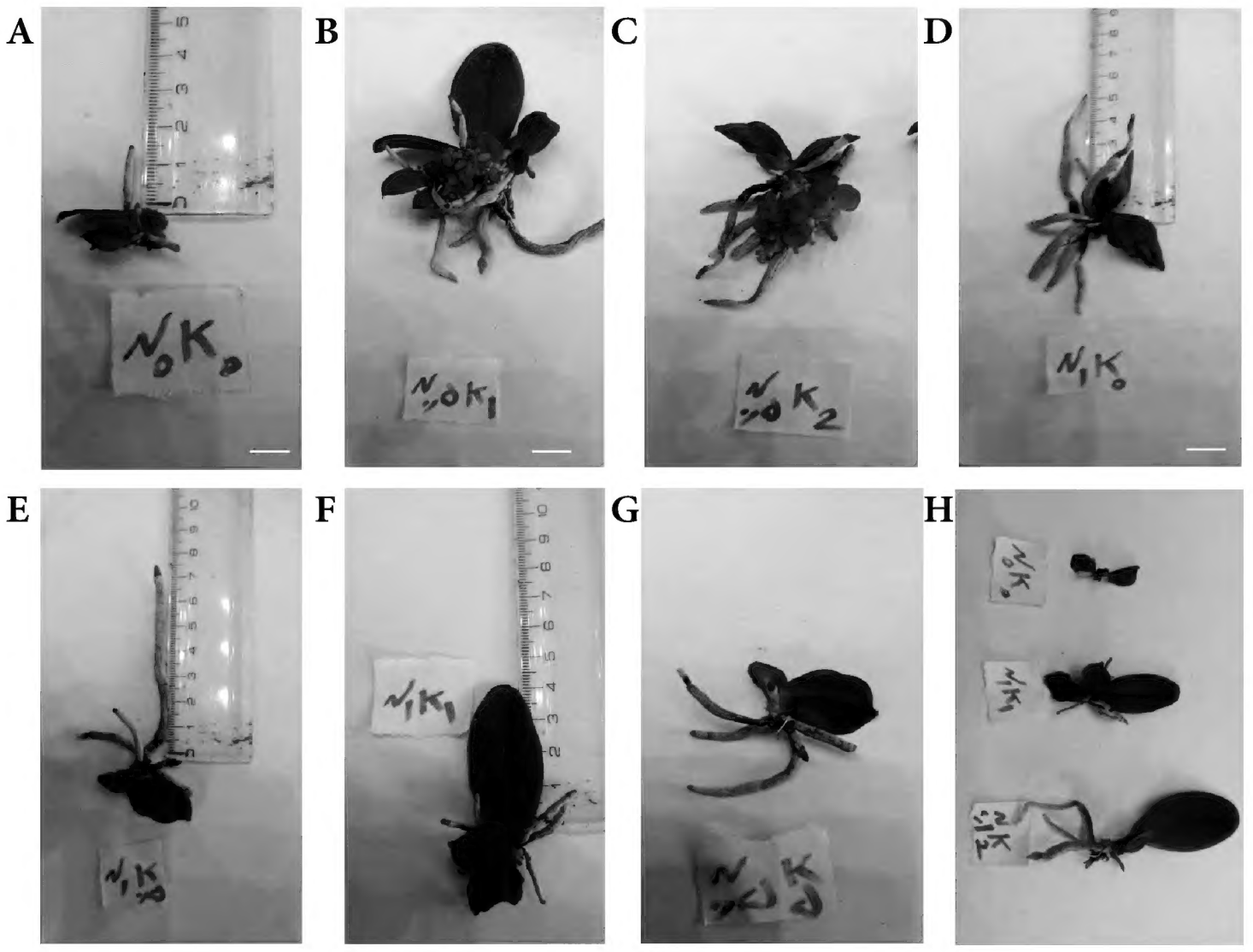

Figure 2. Micropropagation process of *Phalaenopsis circus* using different concentrations of NAA and Kin through organogenesis. Growth and development of leaves and roots from cultured protocorm explants obtained from germinated seeds **A** on control medium **B** on medium enriched with 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} Kin **C** on medium enriched with 0.5 mg l^{-1} NAA and 2.0 mg l^{-1} Kin **D** on medium enriched with 1.0 mg l^{-1} NAA without Kin **E** on medium enriched with 1 mg l^{-1} NAA and 0.5 mg l^{-1} Kin **F** on medium enriched with 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} Kin **G** on medium enriched with 0.5 mg l^{-1} NAA and 5.0 mg l^{-1} Kin, and H) on control medium (up), medium enriched with 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} Kin (middle) and medium enriched with 0.1 mg l^{-1} NAA and 2 mg l^{-1} Kin (down). Scale bar: 10 mm.

Applying 0.5 mg l^{-1} IBA together with 1.0 mg l^{-1} TDZ with 6.33 leaves and 0.5 mg l^{-1} IBA together with 0.5 mg l^{-1} TDZ with 5.33 leaves induced the highest number of leaves as compared to those induced with other treatments and in the absence of PGRs (2.13). Rooting efficiency was satisfactory using IBA at the concentration of 1.0 mg l^{-1} (Table 5). Both root number (5.67) and root length (5.63 cm) in the presence of 1.0 mg l^{-1} IBA were the highest. Root length and root number were lowest in explants grown on PGR-free media.

Ex vitro establishment of plantlets

Well-developed plantlets were transferred to plastic pots for *ex vitro* establishment and acclimatization (Fig. 5). A 100% establishment rate was obtained and plantlets were morphologically identical to the mother plants.

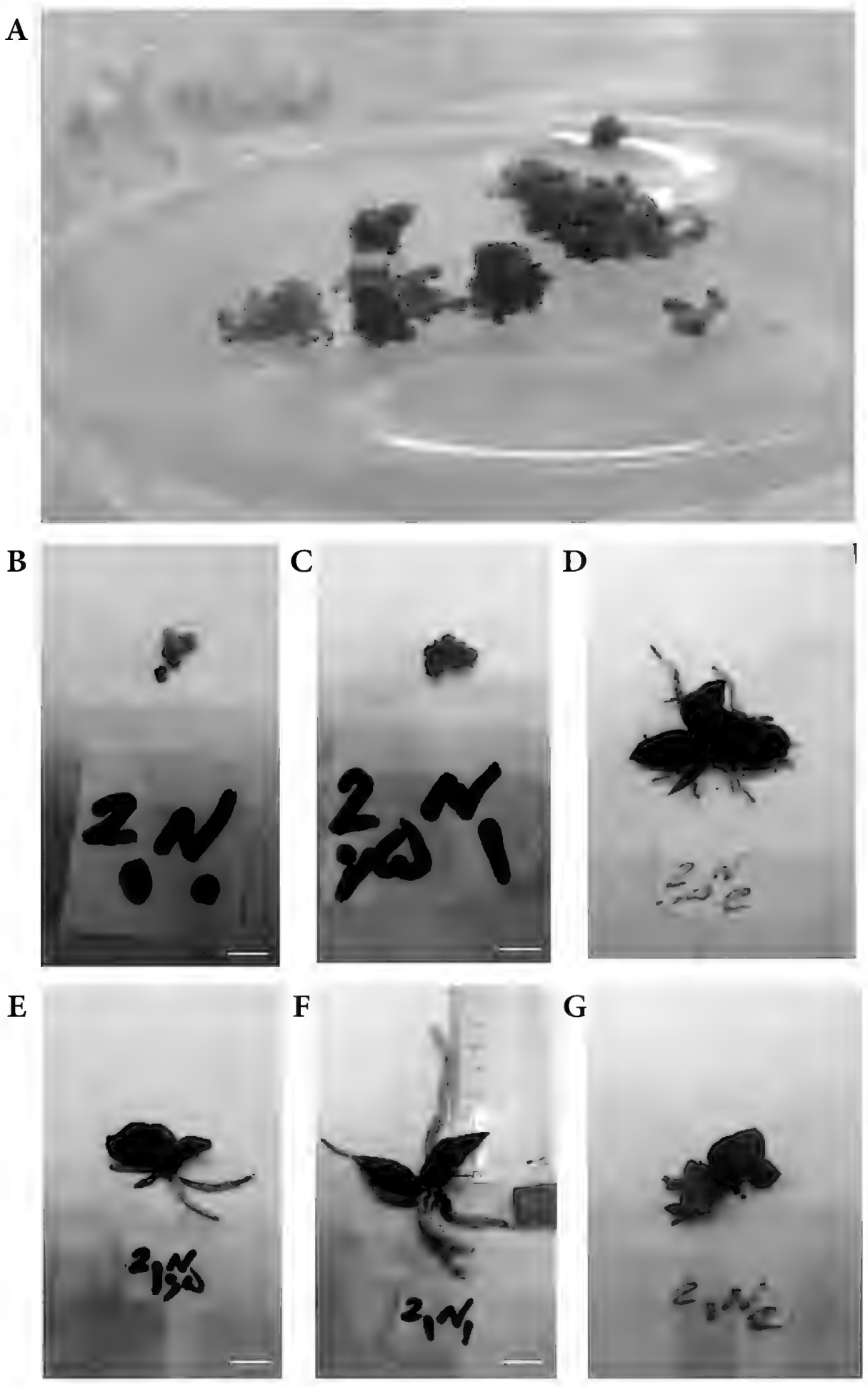

Figure 3. Micropropagation through somatic embryogenesis of *Phalaenopsis circus* using different concentrations of 2,4-D and NAA **A** Somatic embryos produced through inoculation of protocorm on medium enriched with 2,4-D and NAA **B** a somatic embryo on control medium **C** on medium enriched with 0.5 mg l^{-1} 2,4-D and 1.0 mg l^{-1} NAA **D** on medium enriched with 0.5 mg l^{-1} 2,4-D and 2.0 mg l^{-1} NAA **E** on medium enriched with 1.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} NAA **F** on medium enriched with 1.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} NAA, and **G** on medium enriched with 1.0 mg l^{-1} 2,4-D and 2.0 mg l^{-1} NAA. Scale bars: 5 mm (**A, B, C**); 10 mm (**D, E, F, G**).

Table 4. Mean comparison of the effect of different concentrations of 2,4-D and NAA on measured parameters of *Phalaenopsis circus* grown under *in vitro* conditions for somatic embryogenesis.

2,4-D + NAA (mg l^{-1})	Mean comparison	
	Somatic embryo number	Root length (cm)
0.0 + 0.0	6.33c-g	2.3j
0.0 + 0.1	5.67d-g	2.6ij
0.0 + 0.5	5.67d-g	2.77g-j
0.0 + 1.0	6.67c-f	2.87f-j
0.0 + 2.0	5.33efg	4.03bcd
0.01 + 0.0	5.67d-g	2.3j
0.01 + 0.1	4.67fg	2.77g-j
0.01 + 0.5	5.67d-g	2.43ij
0.01 + 1.0	4.67fg	3.1e-i
0.01 + 2.0	5.67d-g	4.5abc
0.1 + 0.0	4.67fg	2.77g-j
0.1 + 0.1	4.33g	3.03e-j
0.1 + 0.5	6.67c-f	2.47ij
0.1 + 1.0	5.67d-g	3.4d-h
0.1 + 2.0	6.33c-g	4.97a
0.5 + 0.0	7.67bcd	2.4j
0.5 + 0.1	12.0a	2.67hij
0.5 + 0.5	9.0b	3.03e-j
0.5 + 1.0	9.0b	2.8g-j
0.5 + 2.0	12.33a	3.53d-g
1.0 + 0.0	7.33b-e	4.7ab
1.0 + 0.1	7.0b-e	3.6def
1.0 + 0.5	8.0bc	3.93bcd
1.0 + 1.0	8.0bc	4.93a
1.0 + 2.0	7.67bcd	3.8cde

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

Discussion

In the present study, induction of callus formation from the protocorms and leaves of *P. circus* did not occur in any of the treatments. This is consistent with the findings for some other orchids like leaf explants of *Paphiopedilum* spp. (Guo et al. 2024). On the contrary, calluses have been successfully induced from the seeds or protocorms of some species of *Paphiopedilum* (Zeng et al. 2013; Guo et al. 2024).

Direct shoot regeneration and multiplication from PLBs and protocorms explants is a successful approach for *in vitro* propagation of orchids (Chugh et al. 2009; Lee and Yeung 2018; Guo et al. 2024), such as *Renanthera imschootiana* (Seeni and Latha 1992), *Cymbidium aloifolium* (Bujarbarua and Sharma 1997; Kaur and Sharma 1997), *Dendrobium aphyllum* (Talukdar 2001), *Habenaria marginata* (Sheelavanthmath and Murthy 2001), *Vanda coerulea* (Roy et al. 2011), *Eulophia nuda* Lindl. (Panwar et al. 2012), *Orchis catasetum* (Baker et al. 2014), *Catasetum pileatum* cv. Alba (Zakizadeh et al. 2019), and *Phalaenopsis amabilis* (Mohammadi et al. 2019).

PLBs production from protocorm culture was influenced by concentration and combination of auxins and cytokinins. In the present investigation, the highest PLB

Table 5. Mean comparison of the effect of different concentrations of IBA and TDZ on measured parameters of *Phalaenopsis circus* plantlets obtained from *in-vitro* cultured TCLs.

IBA + TDZ (mg l^{-1})	Mean comparison			
	Plantlet number	Leaf number	Root length (cm)	Root number
0.0 + 0.0	2.7e	2.13d	1.7g	1.93c
0.0 + 0.01	3.0e	2.33d	2.2fg	2.33bc
0.0 + 0.1	3.33de	2.67cd	2.2fg	2.33bc
0.0 + 0.5	3.67cde	3.0cd	2.7def	3.0bc
0.0 + 1.0	3.0e	3.0cd	2.1fg	3.0bc
0.1 + 0.0	3.33de	3.0cd	2.53def	3.0bc
0.1 + 0.01	3.0e	3.33cd	2.67def	3.0bc
0.1 + 0.1	3.0e	2.67cd	2.2fg	2.0c
0.1 + 0.5	3.67cde	3.0cd	2.8de	3.0bc
0.1 + 1.0	3.67cde	2.33d	2.27efg	3.0bc
0.5 + 0.0	3.67cde	3.33cd	2.53def	5.0a
0.5 + 0.01	3.33de	3.67cd	2.93d	3.0bc
0.5 + 0.1	4.0cde	3.0cd	3.57bc	3.33b
0.5 + 0.5	5.0c	5.33ab	4.13b	2.33bc
0.5 + 1.0	9.0a	6.33a	3.53c	2.0c
1.0 + 0.0	3.67cde	3.67cd	5.63a	5.67a
1.0 + 0.01	4.33cde	3.67cd	4.13b	2.33bc
1.0 + 0.1	4.0cde	4.0bc	3.83bc	3.0bc
1.0 + 0.5	4.67cd	3.33cd	3.93bc	3.0bc
1.0 + 1.0	6.67b	3.67cd	3.83bc	2.67bc

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

number in *P. circus* was obtained by 2,4-D alone and NAA together with TDZ. The combination, type, concentration and the ratio between PGRs plays a critical important role for the formation of shoots, protocorms and PLBs in many orchids (Arditti and Ernst 1993; Bhattacharyya et al. 2016; Kaviani et al. 2017). The most commonly used auxins in orchid culture media are IAA, NAA, IBA, and 2,4-D. On the other hand, Kin, BA, BAP, TDZ, and zeatin are the most commonly used cytokinins in orchid culture media (Yam and Arditi 2018).

Maximum PLB regeneration and the highest root length in *Orchis catasetum* were achieved on media containing both BA and NAA (Baker et al. 2014). The addition of exogenous PGRs in appropriate concentrations promoted shoot multiplication from PLBs (Bhattacharyya et al. 2016). A combination of 1.0 mg l^{-1} Kin and 1.0 mg l^{-1} IBA induced maximum PLB regeneration and the largest number of leaves in *Catasetum pileatum* cv. Alba. Also, the highest rooting frequency was achieved on PLBs grown in medium supplemented with 1.0 mg l^{-1} Kin together with 0.5 mg l^{-1} IBA (Zakizadeh et al. 2019). In another study, a combination of 0.5 mg l^{-1} IBA along with 0.5 mg l^{-1} Kin was suitable for rooting in *Phalaenopsis amabilis* Blume var. Grandiflora (Mohammadi et al. 2019). In the orchid *Vanda coerulea*, a combination of NAA and BAP was found to be optimal for maximum PLB regeneration (Roy et al. 2011).

The current study showed that the lowest number of PLBs was produced on media without PGRs or with low and high concentrations of PGRs. Minimum PLB number was observed on media containing different concentrations of TDZ or Kin, alone.

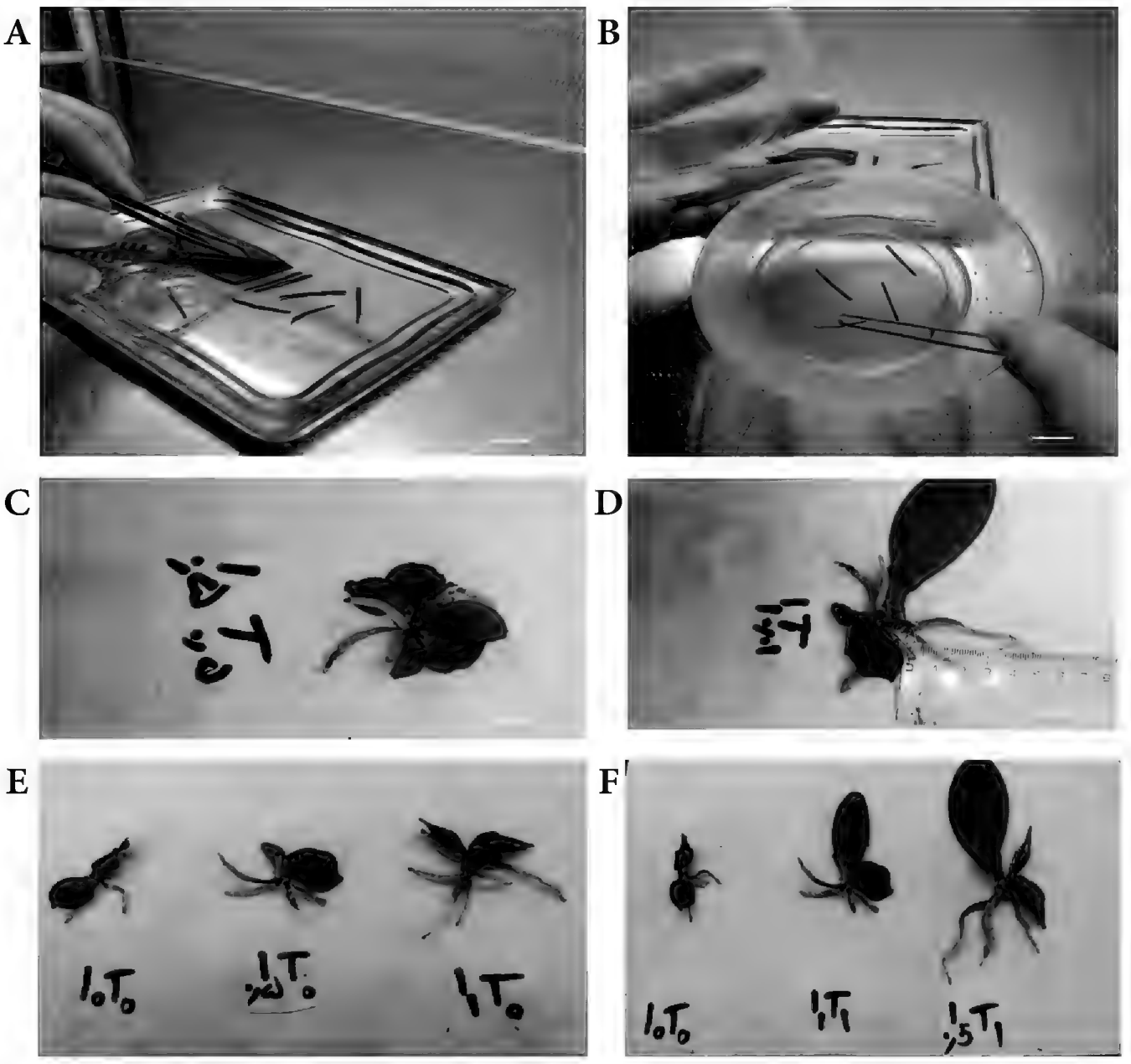

Figure 4. Micropropagation through TCLs of *Phalaenopsis circus* using different concentrations of IBA and TDZ **A** Preparing TCLs from a leaf **B** inoculation of explants on culture medium **C–F** plantlets produced **C** on medium enriched with 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} TDZ **D** on medium enriched with 1.0 mg l^{-1} IBA and 0.01 mg l^{-1} TDZ **E** on media without PGRs (left), enriched with 0.5 mg l^{-1} IBA without TDZ (middle), and enriched with 1.0 mg l^{-1} IBA without TDZ, and **F** on media without PGRs (left), enriched with 1.0 mg l^{-1} IBA and 1.0 mg l^{-1} TDZ (middle), and enriched with 0.5 mg l^{-1} IBA and 1.0 mg l^{-1} TDZ. Scale bars: 10 mm (**A, B, C, E**); 15 mm (**D, F**).

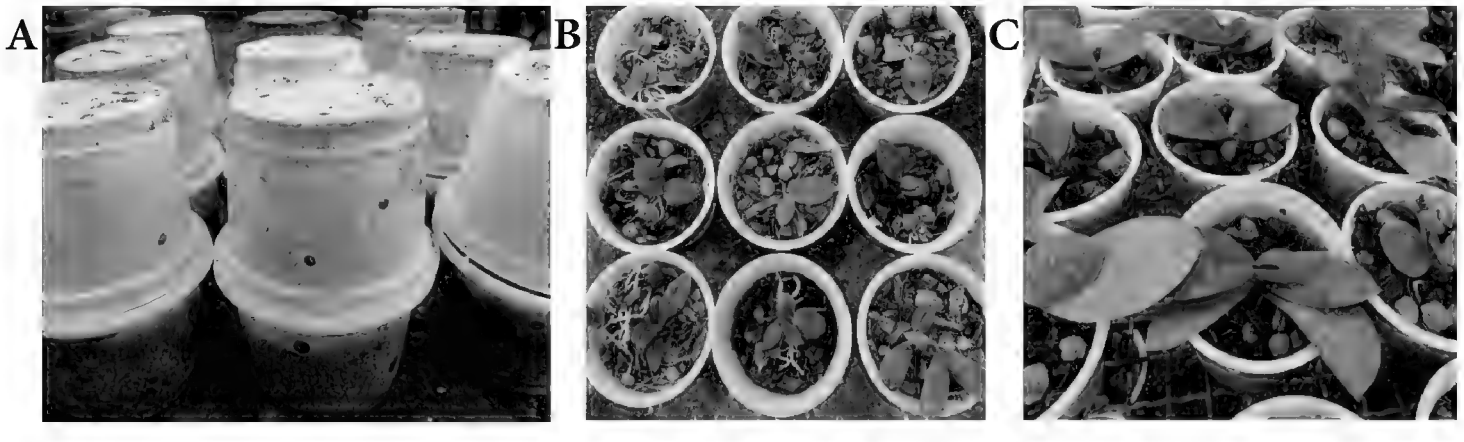

Figure 5. Acclimatization in a greenhouse of plantlets produced *in vitro* and transferred to plastic pots filled with LECA (Light Expanded Clay Aggregate), peat moss, coco peat, charcoal soil, coco chips and perlite in the proportion of 15:10:20:5:30:20% **A** Cultivation of plantlets in plastic pots covered with another plastic pot having several holes **B, C** growing plantlets in pots without cover.

Similar findings were reported in other orchids (Chugh et al. 2009; Panwar et al. 2012; Lee and Yeung 2018). Appropriate formation of PLBs and shoots in *Dendrobium nobile* was performed in medium supplemented with NAA together with BAP, TDZ or meta-topolin (Bhattacharyya et al. 2016). Unlike these reports, some researchers demonstrated that the multiplication of shoots is closely related with the type and concentration of cytokinins used (Amoo et al. 2014; Bhattacharyya et al. 2016; Lee and Yeung 2018). The presence of cytokinins alone promoted optimal shoot proliferation from protocorm explants in some orchids like *Dendrobium nobile* and *C. aloifolium* (Nayak et al. 1997), *C. ensifolium* (Chang and Chang 1998), *Rhynchostylis gigantea* (Van Le et al. 1999), *D. nobile* and *C. aloifolium* (Nayak et al. 2002), and *Dendrobium* (Ferreira et al. 2006). It has been shown that TDZ is the most effective cytokinin for stimulating shoot formation in orchids (Huetteman and Preece 1993; Ket et al. 2004; Martin and Madassery 2006; Zhao et al. 2007; Mahendran and Narmatha Bai 2009). TDZ was the best cytokinin for multiple shoot induction from the protocorm of *Satyrium nepalense* D.Don. (Mahendran and Narmatha Bai 2009). TDZ was first used for *in vitro* propagation of *Phalaenopsis* and *Doritaenopsis* (Ernst 1994). Also, IBA was effective for inducing healthy roots. In *Eulophia nuda* Lindl., maximum PLB formation, shoot multiplication and elongation were obtained on MS medium containing 8.88 μM BA and 4.68 μM Kin after four weeks of culture (Panwar et al. 2012). Luo et al. (2008) showed that 0.50 mg l^{-1} Kin was appropriate for PLB formation of *Dendrobium densiflorum*. BAP alone was better than in combination with NAA for producing the highest number of PLBs in *Oncidium* (Kalimuthu et al. 2007).

The present investigation revealed that the longest roots were induced using both NAA and Kin in combination. Most studies on orchids showed the highest root length and number on media containing an auxin particularly IBA (Chugh et al. 2009; Panwar et al. 2012; Lee and Yeung 2018). Maximum root induction (86.0%) in *Dendrobium nobile* was obtained on half-strength MS medium enriched with 2.0 mg l^{-1} of IBA. The effectiveness of IBA in root induction and growth has been reported for some other orchids such as *Vanilla planifolia* (Giridhar et al. 2001) and *C. pendulum* (Nongdam et al. 2006), too. The main reasons for these differences are the orchid species and cultivars, endogenous PGR levels and type and concentration of exogenous PGRs. In the present study, addition of NAA and TDZ in appropriate concentrations induced plantlet growth and leaf formation from PLBs without callus formation. The major advantage of direct organogenesis without an intervening callus phase is reduction of somaclonal variation (Roy et al. 2011).

SE is an important method of plant regeneration, and several reports have described this technology for some orchids (Chung et al. 2005; Kuo et al. 2005; Chen and Chang 2006). The process of SE is affected by many factors particularly PGRs. In this study, combinations of 2,4-D and NAA were successfully used to induce SE in *P. circus*. There were obvious differences in the number of embryos induction between 2,4-D and NAA. Media containing low concentrations of these PGRs failed to produce embryos. The combination of 2,4-D and other PGRs during SE has been reported for many plants (Yang et al. 2018). Some studies also showed the effectiveness of NAA for SE (Yang et al. 2018). In the present study, NAA did not have a significant effect on SE, when

used alone. TDZ is apparently a useful cytokinin for SE. Direct SE from young leaves of *Oncidium* 'Gower Ramsey' using half-strength MS medium containing 0.3–3.0 mg l^{-1} TDZ was reported (Chen et al. 1999). TDZ promoted direct SE from the epidermal cells and secondary SE from leaf explants of *Phalaenopsis amabilis* (Chen and Chang 2006). Auxins IAA, IBA, NAA, and 2,4-D retarded embryo formation but cytokinins 2-iP, Zt, Kin, BAP and TDZ promoted it in leaf explants of *Dendrobium* cv. Chiengmai Pink. Maximum direct embryo induction was obtained with 18.16 μM TDZ (Chung et al. 2005). Embryogenic calluses were induced from PLBs of *Cymbidium* Twilight Moon 'Day Light' on medium supplemented with NAA or 2,4-D alone or in combination with TDZ. The medium containing the combination of 0.1 mg l^{-1} NAA and 0.01 mg l^{-1} TDZ was optimal for callus formation. PLB formation from callus was obtained through transfer of callus to medium without PGRs. These callus-derived PLBs converted into normal plants on the PGR-free medium (Huan et al. 2004). Clusters of somatic embryos were formed from leaf explants of *Phalaenopsis* 'Little Steve' on half-strength MS medium enriched with BA and TDZ. Kin had no effect on direct embryo induction and 2,4-D retarded the frequency of embryogenesis that was induced by TDZ (Kuo et al. 2005). Therefore, the selection of PGR types, concentrations and combinations is an important criterion for the development of somatic embryos in orchids, as in other plants.

The TCL technique is a simple and effective approach for *in vitro* propagation of orchids. TLCs have been successfully applied for PLB and callus induction in some orchids like *Cymbidium* spp., *Dendrobium* spp., *Aranda*, *Coelogyne*, *Doritaenopsis*, *Paphiopedilum*, *Renanthera*, *Rhynchostylis*, *Spathoglottis*, and *Xenikophyton* (Teixeira da Silva 2013). In the current study, maximum plantlet regeneration was achieved from explants grown on media enriched with 0.5 mg l^{-1} IBA together with 1.0 mg l^{-1} TDZ. In *Dendrobium gratiosissimum*, MS medium containing 2.0 mg l^{-1} Kin developed the highest number of PLBs from thin protocorm sections. Also, the highest PLB production from thin stem sections was achieved from explants cultured in the presence of 5.0 mg l^{-1} Kin together with 1.0 mg l^{-1} NAA (Jaiphet and Rangsayatorn 2010). Direct induction of multiple shoot buds was obtained from thin sectioned leaf explants of *Phalaenopsis* cultured on half-strength MS medium enriched with 9.08 μM TDZ (Myint et al. 2009). In *Aranda*, *Phalaenopsis* and *Cymbidium*, the highest number of PLBs was obtained from TCLs excised from shoot tips (Teixeira da Silva et al. 2007).

Conclusions

The growing popularity of orchids around the world has encouraged propagators and breeders of these valuable plants to develop the orchid flower industry more than ever. Providing effective protocols for their *in vitro* propagation using appropriate techniques, explants, and PGRs is one way to reach this goal. The current study presents effective methods for *Phalaenopsis circus in vitro* propagation, some of which are reported for the first time for this species of orchid. Briefly, of the three auxins used, 2,4-D was found to be the best for PLB induction. Also, of the two cytokinins used, TDZ

was better than Kin for plantlet production. Both organogenesis and SE were proper approaches for induction of shoots and roots and multiplication of *P. circus*. TCLs, one of the most recent techniques for the *in vitro* propagation of selected orchid species have the potential for large-scale commercial multiplication of these valuable orchids.

Acknowledgement

This work was supported by Islamic Azad University, Rasht Branch. The authors would like to thank Dr. Naser Negahdar, Razieh Taghizadeh Andevari and Dr. Shima Seydi for their valuable technical assistance.

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